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Investigating the putative hybrid, *Carex baileyi* × *Carex lurida*

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Abstract

Plants of intermediate proportions and high levels of achene sterility have been observed in sympatric populations of the ubiquitous *Carex lurida* Wahlenb. and the uncommon *C. baileyi* Britt. Species within section Vesicariae are already known to hybridize more frequently than other *Carex* spp., so it was postulated that these sterile, intermediate plants may be hybrids. Using material from three sites in Berkshire County, Massachusetts, genetic sequencing of ndhf and matK chloroplast DNA and nuclear ITS2 markers revealed no consistent differences between the putative hybrids and either of the parent taxa. However, these same data also showed no difference between the parent taxa themselves, suggesting that *C. baileyi* may not be genetically distinct enough to warrant species status, perhaps relegating it to a synonym for *C. lurida.* Alternatively, alternate genetic markers are required to discover their differences. Within Massachusetts, field observations of habitat and gestalt of *C. baileyi* generally support its recognition as a distinct species, but intermediate plants will continue to elude assignment to any one taxon with confidence.

Background

Carex lurida Wahlenb. is a ubiquitous sedge of various wetland habitats throughout the Northeast. Within Massachusetts, *C. baileyi* Britt. is a listed rare species (S3: Special Concern), occurring infrequently in sub-acidic wetlands, typically in the higher elevations of western Massachusetts (Mass. NHESP 2019). *C. baileyi* is frequently sympatric with *C. lurida*, yet hybridization between the two has not been formally reported, possibly due to the broad morphological ranges reported for *C. lurida*, overlap of these morphologies with *C. baileyi*, and the generally subtle differences between the two taxa (Ball & Reznicek 2003).

Several workers familiar with the taxa in question have observed troublesome intermediacy. At one site utilized in this study, the original discoverer encountered difficulty in assigning a positive identification due to morphological intermediacy, noting plants "a bit too fat-looking" for *C. baileyi*, but ultimately settling on "*lurida* towards *baileyi*, or *lurida* × *baileyi*" (Sorrie 1987). Another botanist, working with this same population in recent years, also posited hybridization as a potential explanation for these sterile intermediate plants, which still persist at this site (M.G. Hickler, pers. comm.). Similarly, another sterile intermediate plant found around a beaver pond in New York was presumed to be a hybrid (Reznicek, pers. comm.). Two populations of *C. baileyi* that I discovered in 2015 and 2016 also featured such intermediates, effectively serving as a catalyst for this investigation.

Species within *Carex* section Vesicariae are known to hybridize more frequently than most other sections of *Carex*. Furthermore, hybrids within this section have been reported with greater frequency in the glaciated portions of northeastern North America (Cayouette and Catling 1992). Hybrid plants generally have intermediate morphology relative to the parent taxa, and they exhibit high levels of achene sterility (Reznicek and Catling 1985, Catling et al. 1989, Catling 1993, Catling 1996).

Given the overlapping morphological ranges for spike, perigynium and leaf width dimensions for these taxa, genetic analysis presents as the best tool to investigate putative hybrids between these presumably very closely-related species.

Materials and Methods

Sample

Initial sequencing work on the parent taxa was conducted in spring 2018 using material collected from clear parental taxa identified by leaf width on 29 May 2018. Samples were severed from live, healthy foliage using sterilized scissors, and material was cut such that it fell directly into a collection bag containing silica bead desiccant without any intermediate handling. These plants were flagged in the field for future vouchering and exclusion from subsequent sampling.

Three populations of putative hybrids were sampled, each from Berkshire County, Massachusetts (Table 1). Fresh leaf samples were collected on 30 July 2018, consisting of several square centimeters of leaf tissue from each of the parent taxa and from one intermediate individual, using the same collection protocol noted above. Additionally, samples of leaf tissue from dried specimens, collected in 2015 and 2016, were prepared to supplement the fresh material. Samples were labeled and shipped overnight to the Pringle Herbarium, Burlington, VT, where they were promptly placed in a freezer until analysis. Voucher specimens of reproductive material were collected simultaneously for each plant sampled (Table 1), and specimens were accessioned at MASS in 2019. Voucher specimens of the plants used for initial sequencing were also collected on this date.

Table 1: Specimens collected, locations and voucher information. Leaf sample ID and Collection No. are both included on the physical specimens at MASS for cross-referencing with genetic sequencing data.

Field Identification	Site Name	Town (MA)	Latitude	Longitude	Collection Date	Leaf Sample ID	Collection No.	Voucher Barcode at MASS
Carex baileyi	Busby Trail powerline	Florida	42.6622	-73.0608	7/30/18	FB18-01	PPG0189	430337
Carex baileyi	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	7/30/18	HB18-01	PPG0194	430342
Carex baileyi	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	7/30/18	HB18-02	PPG0193	430343
Carex baileyi	Dalton Fire District, New Windsor Rd.	Hinsdale	42.4681	-73.0832	7/30/18	HB18-03	PPG0195	430341
Carex lurida	Busby Trail powerline	Florida	42.6622	-73.0608	7/30/18	FL18-01	PPG0190	430336
Carex lurida	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	7/30/18	HL18-01	PPG0191	430335
Carex lurida	Dalton Fire District, New Windsor Rd.	Hinsdale	42.4698	-73.0846	7/30/18	HL18-02	PPG0197	430339
Carex lurida	Dalton Fire District, New Windsor Rd.	Hinsdale	42.4681	-73.0832	7/30/18	HL18-03	PPG0196	430340
cf. *Carex lurida x baileyi*	Busby Trail powerline	Florida	42.6622	-73.0608	7/30/18	FX18-01	PPG0188	430334
cf. *Carex lurida x baileyi*	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	7/6/15	HIN01	PPG0182	430356
cf. *Carex lurida x baileyi*	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	8/15/16	HIN08	PPG0183	430347
cf. *Carex lurida x baileyi*	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	8/15/16	HIN09	PPG0184	430348
cf. *Carex lurida x baileyi*	Dalton Fire District, Hinsdale Rd.	Hinsdale	42.4766	-73.0891	7/30/18	HX18-01	PPG0192	430338
cf. *Carex lurida x baileyi*	Dalton Fire District, New Windsor Rd.	Hinsdale	42.4681	-73.0832	8/11/16	HIN07	PPG0186	430345
cf. *Carex lurida x baileyi*	Busby Trail powerline	Florida	42.6622	-73.0608	10/25/16	FLO01	PPG0185	430346

Genetic Analysis

Three genetic markers were sequenced: the nuclear marker ITS2 (330 bp); the chloroplast marker ndhf (1140 bp); and the additional chloroplast marker matK (1220 bp), for a total of 2690 bp.

Extraction: Two fresh samples of *Carex baileyi* and *Carex lurida* were extracted using the adapted Barrington Lab DNA extraction protocol. Four extractions were made from each sample.

Primers: Primers for DNA sequencing are from Gilmour et al. (2013) and Řepka et al. (2014); they are listed in Table 2.

Table 2. Primers used in the DNA analysis of the putative hybrids of *Carex baileyi* and *C. lurida*

Primer Name	5' to 3' sequence	Scale Requested	Purification Requested
matK-1F	CGTCAACAACAATGCTTATATCC	25 nmole	Standard desalting
matK-RL	GCTTTGCCTTGATATCGAAC	25 nmole	Standard desalting
matK-2.5F	TCAATGCTGGRTCCAAGATA	25 nmole	Standard desalting
matK-2.5R	ATATCTTGGARCCAGCATTG	25 nmole	Standard desalting
matK-5R	TTTATGTTTACGAGCCAAAG	25 nmole	Standard desalting
ndhF - A	TATGGTTACCTGATGCCATGGA	25 nmole	Standard desalting
ndhF - B	CCCCATAGAGATATTGAAT	25 nmole	Standard desalting
ndhF - C	TAACAGCATTTTATATGTTTCG	25 nmole	Standard desalting
ndhF - D1	CTATRTAACCRCGATTATATGACCAA	25 nmole	Standard desalting
ITS3i	GCATCGATGAAGAACGTAGC	25 nmole	Standard desalting
ITS4i	GGTAGTCCCGCCTGACCTGG	25 nmole	Standard desalting

PCR: Protocol 1 was taken directly from the papers and protocol 2 was adapted using https://tmcalculator.neb.com/#!/main to determine a different annealing temperature.

matK protocol

1. 94°C for 2 minutes for pretreatment, 40 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 47°C for 60 sec, DNA extension at 72°C for 90 secs, final step at 72°C for 8 mins.
2. 94°C for 2 minutes for pretreatment, 40 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 53°C for 60 sec, DNA extension at 72°C for 90 secs, final step at 72°C for 8 mins.

NDHF protocol

1. 94°C for 2 minutes for pretreatment, 40 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 47°C for 60 sec, DNA extension at 72°C for 120 secs, final step at 72°C for 8 mins.

2. 94°C for 2 minutes for pretreatment, 40 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 61°C for 60 sec, DNA extension at 72°C for 120 secs, final step at 72°C for 8 mins.

ITS protocol

1. 94°C for 2 minutes for pretreatment, 35 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 58°C for 60 sec, DNA extension at 72°C for 50 secs, final step at 72°C for 8 mins.
2. 94°C for 2 minutes for pretreatment, 35 cycles, DNA denaturation at 94°C for 30 secs, primer annealing at 59°C for 60 sec, DNA extension at 72°C for 50 secs, final step at 72°C for 8 mins.

Agarose Gel Analysis:

matK: Both protocols worked, although 47°C was a little brighter on the gel.
NDHF: Both protocols worked, however 61°C was brighter on the gel.
ITS: The protocol using 58°C annealing temperature had only three resolved bands, and there was smearing and one unresolved band near the top of the gel. The second protocol worked at 59°C for all samples.

Sequencing:

Samples were sent for sequencing through Genewiz at the New Jersey facility. Sequences were analyzed on the program Geneious. Contigs were assembled for the three markers. Sequences were blasted in genbank to confirm their identity. All matched sequences for the appropriate markers in *Carex* species.

Results

No variation was observed in the chloroplast DNA markers, neither between the putative hybrids and the parent taxa nor between the parent taxa themselves. ITS2 was found to vary at three sites, but this variation occurred within taxa and thus cannot be used to differentiate between taxa.

Discussion

Carex baileyi is morphologically quite similar to *C. lurida*, and the two taxa are presumably more closely related than they are to other *Carex* spp. The lack of variation found in these genetic sequencing data reinforces the presumption of this close relation. Although an insufficient amount of variation was an expected potential outcome of this investigation, the total absence of variation between the parent taxa warrants further commentary.

In a broad study of *Carex* section Racemosae using combined ITS2 and cpDNA sequences, Gebauer et al. (2015) commonly reported a minimum of 5-nucleotide inter-species differences for species pairs for a 4700bp dataset, and they commonly retrieved intraspecific variation. Although our dataset was smaller (1140 bp), the rate of variation (5 differences per 4700 bp, or >1 difference per 1000 bp of sequencing data) in this study suggests that it is improbable that we would fail to detect any differences between the two parent taxa, unless they were in fact taxonomically identical. Yet we cannot conclude this definitively, since further sequencing may plausibly detect some variation not captured by this analysis.

In western Massachusetts, most populations of *Carex baileyi* appear to be morphologically distinct, with very narrow carpellate spikes and narrower leaves than sympatric populations of *C. lurida*. A trained eye can readily assign the majority of plants to one taxon or the other. Troublesome plants of intermediate proportions present a singular problem, especially when attempting to accurately survey populations of the state-listed *C. baileyi*. It was the hope of this study to establish definitively the existence of hybrid plants, and to provide a basis for identifying such plants in the field. Although this objective was not met, this study has helped bolster the general impression that *C. baileyi* and *C. lurida* are very closely related, and leaves to future investigations to tease out the phylogenetic origins of these seemingly distinct taxa.

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